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# Multiscale imaging of neurons grown in culture: From light microscopy to cryo-electron tomography

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**Abstract**

Cryo-electron tomography (cryo-ET) allows the visualization of supramolecular architecture in cells preserved in a close-to-physiological state. In order to supplement the structural information obtained by cryo-ET with the functional state of the molecules involved based on fluorescent labeling we developed a method of correlating light microscopy and cryo-ET. This method is suitable for investigating complicated cellular landscapes such as mature neurons grown in culture. It has the advantage that a correlation is obtained without exposing a feature of interest to additional electron irradiation, and that it does not rely on visual recognition of features. Different modes of correlation are presented here: a feature identified on a light microscopy image is used to guide the cryo-ET investigation, and cryo-tomograms are correlated to light microscopy images. Cryo-tomograms of a neuronal synapse and of an isolated presynaptic terminal are shown as examples of the correlative method. The correlation method presented here can be expected to provide new insights into the structure–function relationship of supramolecular organization in neurons.


*Keywords:* Cryo-electron tomography; Correlative microscopy; Neuronal cultures; Endocytosis; Synapse

## 1. Introduction

Cryo-electron tomography (cryo-ET) combines the potential of three-dimensional (3D) imaging at molecular resolution with a close-to-life preservation of biological samples. Rapid freezing followed by the investigation of the frozen-hydrated samples avoids the artifacts caused by chemical fixation and dehydration procedures. Furthermore, the biological material is observed directly, without heavy metal staining, avoiding artifacts caused by the unpredictable accumulation of staining material (Baumeister, 2002; Al-Amoudi et al., 2004b). A problem in cryo-ET is the high sensitivity of ice-embedded specimens to electron irradiation. The automated acquisition of electron tomograms helps to keep the total exposure during the recording of a cryo-tomogram within acceptable limits (Koster et al., 1997). However, minimization of the electron dose used during the search for a feature of interest in cryo-ET remains an important issue that needs to be addressed.

Light microscopy (LM) has been correlated to conventional electron microscopy (EM) in order to obtain higher resolution information about structures detected in LM (Nakata et al., 1998; Ahmari et al., 2000). Combinations of immuno-labeling with electron-dense markers and fluorescent labeling have been used for detection of molecules at higher resolution than LM can provide (Mironov et al., 2001; Gaietta et al., 2002). These methods are effective on a cellular scale, however, they either rely on an extensive search by EM, thereby

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increasing the cumulative electron dose, or on the presence of heavy metals, which renders them unsuitable for studies on a molecular scale.

Cryo-ET of whole cells has the advantage that supramolecular architecture can be studied in unperturbed cellular environments (Medalia et al., 2002; Lucic et al., 2005a; Murphy et al., 2006). The cryo-preparation of adherent cells, such as neuronal cells, is more challenging than the cryo-preparation of cells in suspension. Recently, two different approaches were taken to investigate neurons in a frozen-hydrated state: cryo-sections of organotypic neuronal cultures were studied by cryo-EM (Zuber et al., 2005), and cryo-ET was used for the investigation of neurons in culture at an early developmental stage (Garvalov et al., 2006). Studying mature neuronal cultures by cryo-ET adds another layer of complexity arising from the presence of cell bodies together with many long and highly interconnected neuronal processes. This makes it difficult to locate features of interest and a prolonged search can contribute substantially to the total electron dose. We circumvented this problem by using light microscopy to assist a cryo-ET search and to provide cues for identification of larger cellular structures, thus establishing a correlative light and cryo-ET approach. The purely software-based approach, presented in this paper, differs from a correlative procedure developed by Sartori et al. (2007) in that it requires minimal extra electron dose to be spent on a feature of interest and is therefore consistent with the requirement of minimizing the electron dose. Our procedure does not rely on a visual recognition of features, and so it can be reliably applied to mature neuronal cultures that often show dense networks of interconnecting neuronal processes. Cryo-tomograms of a neuronal synapse and of an isolated presynaptic terminal are shown as examples of the correlative method. We also show that this correlative approach can be used to supplement structural information at the molecular level obtained by cryo-ET with functional information derived from fluorescent labeling.

## 2. Materials and methods

### 2.1. Neuronal cultures

Gold finder EM-grids (type NH2A by Plano, Wetzlar, Germany) coated with Quantifoil (TM) R2/2 were sterilized in ethanol for 10 min, then washed in $H_2O$, and transferred to culture dishes. Both grids and dishes were coated with 1 mg/ml poly-L-lysine (in water) for 1 day, washed in D-MEM medium (Gibco 61965), and placed in D-MEM with B27 supplement prior to plating. Whenever a liquid had to be removed completely from a dish during washing and medium exchange steps, grids were transferred into another dish containing the appropriate liquid in order to prevent grids from drying. Sidewards submersion of grids into the media greatly reduced the risk of ablation of the sensitive grid coating.

Primary hippocampal neuronal cultures were dissected from E18 Sprague–Dawley rats in accordance with the procedures accepted by Max-Planck Institute for Neurobiology, and dissociated as described previously (Goslin and Banker, 1991). Briefly, hippocampal neurons were dissected from embryos and were dissociated after incubation for 30 min in an EDTA/trypsin solution (0.25% trypsin, 1 mM EDTA). After washing with D-MEM and 10% FBS, neurons were plated on the poly-L-lysine coated dishes and EM grids (kept in the dishes). Cultures were kept at 37 °C in 5% $CO_2$ and investigated at 10–15 days in vitro (DIV). In some cases (tomogram shown in Fig. 3) the cultures were treated with 50 ng/ml BDNF (Chemicon) for 24 h before freezing.

### 2.2. Fluorescent labeling and live light microscopy

Cultured neurons grown on EM grids were first labeled for 90 s with 15 μM FM1-43 equivalent (synaptogreen C4, Sigma) in HBSS solution (Gibco 14025050) with the addition of 50 mM KCl, 2 mM $CaCl_2$ (3.3 mM $Ca^{2+}$ total), and 10 μM CNQX (Tocris). This step was followed by 60 s incubation in 15 μM FM1-43 equivalent in HBSS, at 30–35 °C to allow time for the completion of endocytosis. Depolarization-induced FM1-43 unloading was done for 60 s in HBSS solution with the addition of 50 mM KCl, 2 mM $CaCl_2$, and 10 μM CNQX.

The grids were imaged in HBSS in glass-bottom culture dishes (MatTek P35G-1.5-7-c-grid) mounted in a chamber at 35 °C with a Zeiss Axiovert 200M inverted light microscope equipped with motorized stage and using a Zeiss air 10× Plan-Neofluar Ph1 objective with 0.3 N.A., a Zeiss air 20× Achroplan Ph2 objective with 0.4 N.A., and a fluorescence filter set (Zeiss) for FM1-43 (470/40 nm bandpass excitation, 510 nm long-pass beamsplitter, 540/50 nm bandpass emission). In order to compensate for the fact that grids are usually slightly bent and to ensure that the whole grid is imaged in focus, several images comprising a z-stack were taken at each position. Typically, 10–15 images with 5 μm z-increment at 20× were taken. Images covering a whole grid and z-stacks were recorded using Mosaix and z-stack modules of Zeiss Axio Vision 4.1 software. In-focus information was manually extracted from individual z-slices and individual images were combined to show a wider area using TNImage (free software distributed under the GNU public license, http://brneurosci.org/tnimage.html) and GIMP (free software developed by The GIMP Team and distributed under the GNU public license, http://www.gimp.org/) image manipulation software.

### 2.3. Cryo-electron microscopy and tomographic reconstruction

BSA tracer 15 nm gold (Aurion) was repeatedly spun with a tabletop centrifuge at 13,000 rpm (15–30 min total), the supernatant was discarded and the soft pellet